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Interaction of the Paf antagonist WEB 2086 and its hetrazepine analogues with human platelets and endothelial cells

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1 Intact platelets and confluent human umbilical vein endothelial cells bound [^{3}H]-Paf-acether (platelet activating factor, [^{3}H]-Paf) at 20°C in the presence of 0.25% (w/v) bovine serum albumin (BSA).

2 [^{3}H]-Paf binding to platelets was inhibited in a concentration-dependent manner by WEB 2086. An excess of WEB 2086 indicated the presence of specific, saturable Paf binding which reached a maximum of 28.3 ± 3.7 fmol [^{3}H]-Paf per 5×10^7 platelets. In platelets, different hetrazepines (WEB 2098, 2105, but not 2118) also inhibited [^{3}H]-Paf binding in a concentration-dependent manner.

3 WEB 2086 partially displaced platelet-bound [^{3}H]-Paf in a concentration-dependent manner reaching a plateau at 400 nM WEB 2086. No further displacement was observed when WEB 2086 and an excess of unlabelled Paf were added together.

4 The hetrazepines inhibited platelet aggregation. Platelet aggregation IC_{50} values correlated well with the IC_{50} values of the hetrazepines against [^{3}H]-Paf binding ($r^2 = 0.99$). WEB 2086 shifted the Paf dose-response curve rightwards in a parallel manner. Tested against platelet aggregation the pA_2 obtained for WEB 2086 was 7.9.

5 WEB 2086 inhibited [^{3}H]-Paf binding to endothelial cells in a concentration-dependent manner. WEB 2086 also inhibited the Paf-mediated cytosolic calcium increase in endothelial cells with an IC_{50} value of 23.1 ± 10.4 nM as compared with an IC_{50} of 21.6 ± 10.4 nM WEB 2086 for platelet aggregation.

6 These results demonstrate an inhibition of [^{3}H]-Paf binding to platelets and endothelial cells by different hetrazepines, most probably at the Paf receptor level.

Introduction

Paf-acether (Paf, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (Benveniste *et al.*, 1972; 1979; Demopoulos *et al.*, 1979; Blank *et al.*, 1979) exhibits potent inflammatory and hypotensive properties (Vargaftig *et al.*, 1981; Pinckard *et al.*, 1982; Chung & Barnes, 1988). Paf activates platelets independently of the cyclo-oxygenase pathway via a receptor-dependent mechanism (Cazenave *et al.*, 1979; Valone *et al.*, 1982; Korth *et al.*, 1986). Intact washed human platelets do not metabolise Paf, but when platelets are activated or lysed they release their Paf-degrading cytosolic acetylhydrolase (Korth *et al.*, 1988a). Binding sites or receptors for Paf have been reported in platelets (Valone *et al.*, 1982; Korth & Benveniste, 1987), neutrophils (Valone & Goetzl, 1983), macrophages (Lambrecht & Parnham, 1986), membrane preparations of human lung tissue (Hwang *et al.*, 1985) and recently in human cultured umbilical vein endothelial cells (Korth *et al.*, 1987).

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In the latter work, binding sites for Paf on endothelial cells were shown to display a higher affinity than those in platelets.

Several putative antagonists of Paf have recently been described, such as CV 3988 (Terashita *et al.*, 1983), Kadsurenone (Shen *et al.*, 1985), BN 52021, a chemically-defined extract from *Ginkgo biloba* (Braquet, 1984; Nunez *et al.*, 1986; Korth & Benveniste, 1987) and the Ginkgolide-mixture BN 52063 (Guinot *et al.*, 1986; Korth *et al.*, 1988b). The Ginkgolides specifically inhibit platelet aggregation, produce a shift in Paf concentration-response curves and displace labelled Paf from platelets in a concentration- and time-dependent manner suggesting competition at the binding site (Korth *et al.*, 1988b). Certain calcium channel antagonist drugs also inhibit Paf binding (Wade *et al.*, 1986).

Another class of Paf antagonists has recently been proposed. Triazolo-benzodiazepines such as alprazolam and triazolam and triazolo-thieno-1,4-benzodiazepines such as brotizolam inhibit Paf-induced platelet activation (Kornecki *et al.*, 1984; Chesney *et al.*, 1987) and these *in vitro* and *in vivo* effects are dissociated from their CNS activity (Casals-Stenzel & Weber, 1987; Casals-Stenzel, 1987a; Casals-Stenzel *et al.*, 1987b). One specific Paf-antagonist derived from triazolodiazepines, WEB 2086, inhibited Paf-induced platet aggregation (Weber *et al.*, 1986), endotoxin shock (Casals-Stenzel, 1987b), anaphylaxis (Casals-Stenzel, 1987c) and Paf binding to human platelets and endothelial cells (Korth *et al.*, 1987).

In various organs Paf triggers vascular effects such as vasoconstriction and acute oedema formation which are inhibited by Paf receptor antagonists (Benveniste *et al.*, 1983; Guinot *et al.*, 1986; Hwang *et al.*, 1986; Casals-Stenzel *et al.*, 1987a). Several reasons argue for these vascular Paf effects being directly mediated by endothelial cells. Firstly, Paf elicits vascular effects even in the absence of blood cells (Casals-Stenzel, 1987a); secondly, endothelial cells possess Paf receptors with a higher affinity than those in platelets (Korth *et al.*, 1987) and thirdly, Paf receptor antagonists specifically inhibit vascular effects *in vivo* and *in vitro* (Guinot *et al.*, 1986; Casals-Stenzel *et al.*, 1987a; Hwang *et al.*, 1986). The shape change, the Ca^{2+} flux and the release of prostacyclin from endothelial cells in response to Paf (Bussolino *et al.*, 1985; Brock & Gimbrone, 1986; D'Humières *et al.*, 1986; Grigorian & Ryan, 1987; Hirafuji *et al.*, 1988), as well as the Paf-mediated adherence of inflammatory cells (McIntyre *et al.*, 1986; Kimani *et al.*, 1988), could explain the important pathophysiological role of the mediator in allergy and inflammation (Chung & Barnes, 1988; Arnoux *et al.*, 1988). Therefore we investigated the antagonism of Paf receptors in human platelets and endothelial cells using radioligand binding assays and, in the latter, increase in cytosolic Ca^{2+}. We describe in platelets the comparative characteristics of the triazolo-thieno-benzodiazepines WEB 2086, 2098, 2105 and 2118 as antagonists of Paf and of [^{3}H]-Paf binding.

Methods

Preparation of washed human platelets

Platelets were prepared as described previously (Lalau Keraly *et al.*, 1984 modifed by Korth *et al.*, 1988b). For binding studies platelets were incubated in Tyrode buffer containing Aspegic (0.1 mM) for 30 min before the last of three washing procedures.

After the last centrifugation, platelets (1×10^9 ml^{-1}) were kept in acid:citrate:dextrose anticoagulant-free Tyrode buffer (pH 6.4) in the presence of 0.25% bovine serum albumin (BSA) (Korth *et al.*, 1986). The platelet count in the solution was adjusted to 1×10^9 ml^{-1} as determined with a Coulter Counter.

Platelets for aggregation studies were prepared either by the method of Mustard *et al.* (1972) or that described by Korth *et al.* (1988b). The former method was used when the release reaction was studied. Platelets from 150 ml of blood obtained from healthy donors were labelled in the first washing fluid with 2 μCi of [^{14}C]-5-hydroxytryptamine (5-HT). The platelets were finally resuspended at 5×10^8 ml^{-1} in modified Tyrode solution (pH 7.35) at 37°C and containing 0.35% BSA, 2 mM Ca^{2+}, 1 mM Mg^{2+}, 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), apyrase (0.7 μl ml^{-1} of platelet suspension), and 5 μM imipramine.

Platelet aggregation and release reaction in the presence of antagonists

Platelets were incubated in the aggregometer cuvette with WEB 2086 and 2098 (1, 5, 10, 50, 100, 1000 nM, final concentrations), 2105 or 2118 (0.1, 0.5, 1, 5 μM) or with their solvents. After stirring for 30 s at 37°C, fibrinogen was added and platelets were submitted to the 5-HT release and the aggregation procedures in the presence of Paf (0.66 ± 0.08 μM) or ADP (2.5 μM). After 3 min, platelets were pelleted at 11.000 *g* for 1 min in an Eppendorf centrifuge and the radioactivity in 100 μl of supernatant was counted under standard conditions in ACS scintillant.

The antagonism of Paf-induced aggregation was studied by incubating platelets for 30 s at 37°C in the presence of different concentrations of WEB 2086

(11, 22, 33, 44, 88, 100 and 500 nM, final concentrations) before constructing a concentration-response curve to Paf. Control experiments were performed in the presence of drug vehicle alone. Schild plots were constructed as described by Arunlakshana & Schild (1959).

[^2H]-Paf binding studies in intact human platelets

The platelet suspension was diluted (1:10, v/v) in pH 7.4 Tyrode buffer supplemented with 1.3 mM Ca^{2+} and 0.25% BSA before binding and displacement studies which were performed at 20°C. Platelets were separated by vacuum filtration through Whatman GF/C filters (Ferrière, France). The filter-associated radioactivity in the absence of platelets was subtracted from that measured in the presence of platelets to derive the total binding to cells.

Increasing concentrations of all four hetrazepines (4, 10, 20, 40, 200, 400 nM, 1, 2 μM) were incubated for 30 min together with both platelets and 0.065 nM [^{3}H]-Paf. Platelet-bound radioactivity in the presence of hetrazepines was expressed as the percentage inhibition of total platelet-bound [^{3}H]-Paf.

To establish the extent of specific, saturable Paf binding, platelets (5×10^7 in 500 μl) were incubated with increasing concentrations of [^{3}H]-Paf (0.65–6.53 nM) in the presence of either WEB 2086 (2 μM) or 0.2% (v/v) ethanol vehicle for 30 min. The incubation was performed in pH 7.4 Tyrode buffer in the presence of 0.25% BSA and 1.3 mM Ca^{2+}. Binding was expressed as fmol [^{3}H]-Paf bound per 5×10^7 platelets. Non-specific binding to the cells was defined as that not inhibited by 2 μM WEB 2086. The specific binding was defined as the difference between total and non-specific binding.

In a second type of binding experiment, displacement of platelet-bound [^{3}H]-Paf by WEB 2086 was investigated. Platelets (5×10^7 in 450 μl pH 7.4 Tyrode buffer containing 1.3 mM Ca^{2+} and 0.25% BSA) were preincubated for 15 min with 50 μl 0.65 nM [^{3}H]-Paf before addition of 1 μl WEB 2086 (4, 40, 400, 2000 nM) for different time periods. In other experiments WEB 2086 alone, unlabelled Paf (50 nM) alone, WEB 2086 (2000 nM) together with unlabelled Paf (50 nM) or WEB 2098, 2105 and 2118 alone (40 nM) or vehicle alone were added for 30 min to the prelabelled platelets. Platelet-bound radioactivity in the presence of hetrazepines and/or unlabelled Paf was calculated as a percentage of total platelet-bound radioactivity before the additions.

[^3H]-Paf degradation by platelets in the presence and absence of hetrazepines

Platelets (4×10^7 in 400 μl pH 7.4 Tyrode buffer containing 0.25% BSA and 1.3 mM Ca^{2+}) were incubated at 20°C or 37°C with 0.65 nM [^{3}H]-Paf for 30 min. The suspension was then filtered and the filters, in 400 μl water (1 v), were then extracted in 1 ml of dichloromethane/methanol (1:2, v/v). Dichloromethane and water containing 2% acetic acid (1:1, v/v) were added after 24 h extraction at 4°C. The dichloromethane phase was recovered and the water phase was washed with dichloromethane (1 v) three times. Dichloromethane extracts were submitted to high performance liquid chromatography (h.p.l.c.) (Benveniste *et al.*, 1979). All water phases were also counted to exclude the abnormal presence of radioactivity. Using appropriate authentic markers, a retention time of 3–7 min was defined as void volume, 11–13 min as alkyl-acyl-(long-chain)-*sn*-glycero-3-phosphocholine (AAGPC), 18–25 min as Paf and 28–33 min as lyso-Paf. Radioactivity of different fractions was calculated as a percentage of the radioactivity in all fractions after subtraction of background values.

[^3H]-Paf binding to endothelial cells

Endothelial cells were isolated from umbilical cord veins according to the method of Jaffe *et al.* (1973) with modifications (Hirafuji *et al.*, 1987). In order to remove culture medium (Ham's F-12 plus 10% heated inactivated foetal calf serum) confluent endothelial cells were washed twice with pH 6.4 Tyrode buffer containing 0.25% BSA and 900 μl of the same buffer adjusted to pH 7.4 and containing 1.3 mM Ca^{2+}, 100 μl 0.65 nM [^{3}H]-Paf with different concentrations of WEB 2086, or its vehicle, added. After 30 min at 20°C, free radioactivity was washed twice from confluent endothelial cells with pH 6.4 Tyrode buffer containing BSA, and then once with cold isotonic saline. Endothelial cells were detached by adding cold isotonic saline containing 5 mM EDTA, followed by incubation at 4°C for at least 30 min. The detached cells were filtered from the medium under vacuum. Incubation buffer was filtered to avoid loss of cells during the washing procedure after the binding period. Filters were washed twice with 10 ml cold Tyrode buffer and radioactivity bound to filters was counted under standard conditions in PCS scintillant. Filter-bound radioactivity in the absence of cells was subtracted from radioactivity measured in their presence. The inhibitory effects of different concentrations of WEB 2086 (1–1000 nM) were tested and calculated in fmol [^{3}H]-Paf per $5.2 \pm 0.15 \times 10^5$ endothelial cells.

Measurement of the increase of cytosolic calcium in endothelial cells and its inhibition by WEB 2086 was performed as previously described (Hirafuji *et al.*, 1988). Endothelial cells were grown to confluence onto coverglasses and continuously superfused (1 ml min^{-1}) with Hank's balanced salt solution

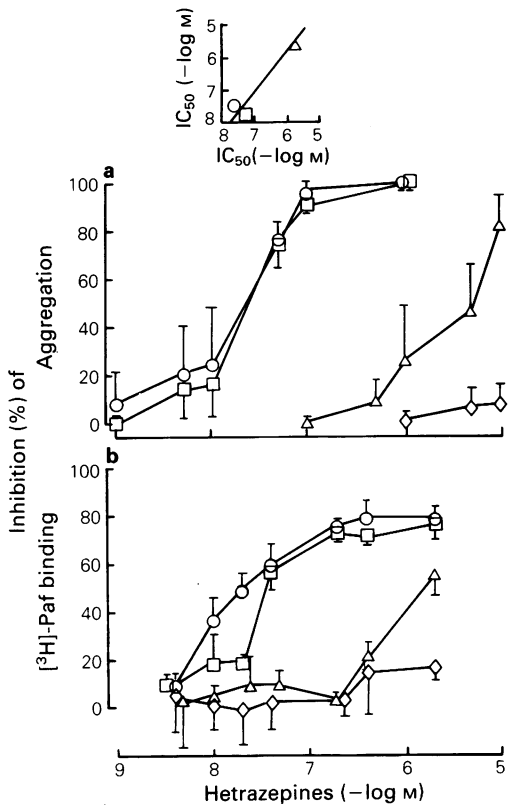

Figure 1 Inhibition by WEB 2086 (○), 2098 (□), 2105 (△) and 2118 (◇) of platelet aggregation in response to Paf (a) and, after 30 min incubation, [^{3}H]-Paf binding to human platelets (b). Each point represents the mean ± 1 s.d. (n = 3–4) inhibition of maximal aggregation or binding; s.d. shown by vertical bars. Inset: correlation between inhibitory hetrazepine effects (IC_{50} in −log M) obtained from dose-response curves (a, b) on Paf-induced platelet aggregation (ordinate scale) and binding of 0.065 nM [^{3}H]-Paf (abscissa scale). Correlation coefficient (r^2) = 0.99.

(HBSS, pH 7.4) containing 10 mM HEPES and 0.1% BSA and incubated for 30 min with 5 μM fura 2/AM. After loading, excess fura 2/AM was removed by washing. When appropriate, cells were superfused with WEB 2086 for 3 min before and during the stimulation.

Confluent endothelial cells were incubated at 20°C for 30 min with 0.65 nM [^{3}H]-Paf under binding conditions. Free radioactivity was carefully washed from confluent endothelial cells as described above. Cells were then detached by freezing and thawing at −20°C three times and the whole cell suspension was extracted as described above in dichloromethane/methanol (1:2, v/v) for h.p.l.c.

Drugs and buffers

Tyrode buffer comprised (in mM): NaCl 137, KCl 2.58, $NaHCO_3$ 11.9, $MgCl_2$ 1.0, NaH_2PO_4 0.41, dextrose 0.5 and HEPES 5.0. Acid citrate dextrose (ACD) comprised citric acid (0.8%), trisodium citrate (2.2%) and glucose (2.45%); citric acid (0.15 M) (all from Merck-Darmstadt, F.R.G). Aspirin was used as its lysine salt (Aspegic, Egic Laboratory, Amilly, France). The following were obtained and prepared as indicated: fatty acid-free BSA, imipramine, collagenase and ADP were from Sigma Chemical Co, St. Louis, MO, U.S.A. [^{3}H]-Paf, (1-O-[^{3}H]-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) sp. act. 80 Ci mmol^{-1}, [^{14}C]-5-hydroxytryptamine-3-creatinine sulphate, sp. act. 50 Ci mmol^{-1} and PCS or ACS scintillation fluids were from Amersham International plc, Aylesbury, Bucks, U.K. Culture media were from Hyclone, Logan, Utah, U.S.A and laboratory ware from Falcon, Oxnard, CA, U.S.A. Paf, 1-octacecyl-2-acetyl-*sn*-glycero-3-phosphocholine and its enantiomer (Bachem, Bubendorf, Switzerland) were dissolved in saline containing 0.25% BSA. WEB 2086, 2098, 2105 and 2118 (3-(4-(2-chlorophenyl)-9-methyl-; -9-cyclopropyl-; 3-(4-(2-chlorophenyl)- and -9-cyclohexyl-6H-thieno-(3,2-f)(1,2,4)triazolo-(4,3-a)(1,4)diazepin-2yl)-1-(4-morpholinyl)-1-propanone (Boehringer-Ingelheim, Ingelheim, F.R.G.) were dissolved freshly each day either in water by adding 0.1 N HCl with ultrasonication, or in ethanol. Apyrase from potatoes was used in the platelet-suspending media at a concentration that converted 0.25 μM ATP to AMP within 120 s at 37°C. Human fibrinogen (grade L, AB Kabi, Stockholm, Sweden) was pretreated with diisopropylfluorophosphate and partially purified. Trypsin-EDTA and Hank's balanced salt solution (Gibco, Paisley, Scotland), and specific antiserum to human factor VIII (Nordic Immunology, Silburg, the Netherlands). Acetic acid was from UCB, Leuven, Belgium. Fura 2/AM was from Dojin (Tohoku, Japan).

Data analysis

All results are given as mean ± 1 s.d. of 3–4 experiments, except when indicated.

Results

Platelet aggregation and 5-HT release

Preliminary studies showed that platelet aggregation IC_{50} values for the antagonists yielded similar values whatever the method used for platelet preparation. WEB 2086, 2098 and 2105 all inhibited, in a concentration-dependent manner, platelet aggre-

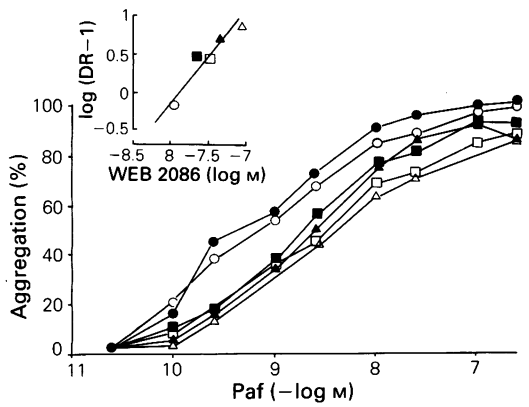

Figure 2 Concentration-dependent aggregation of platelets by Paf in the absence (●) and in the presence of WEB 2086. Platelets were incubated 30 s at 37°C with 11 nM (○), 22 nM (■), 33 nM (□), 44 nM (▲) or 88 nM (△) WEB 2086 prior to aggregation. Each point represents the mean of six experiments. Inset: results from Figure 2 calculated by Schild plot analysis. Each point represents the mean of 6 experiments.

gation induced with 0.66 ± 0.08 μM Paf (Figure 1a). WEB 2105 showed only weak, and WEB 2118 no inhibitory potency. The inhibitory profile for all hetrazepines was the same after preparation in either of the vehicles (results not shown). The 50% inhibitory concentrations (IC_{50}) for platelet aggregation of WEB 2086, 2098 and 2105 were 21.6 ± 10.4 nM, 25.6 ± 4.5 nM and 1.19 ± 0.27 μM respectively (Figure 1a). Similar results were obtained in the presence of 0.25% BSA using the method of Lalau Keraly *et al.* (1984). Platelet aggregation induced by 25 μM ADP was not inhibited by 1 μM WEB 2086 since 72%

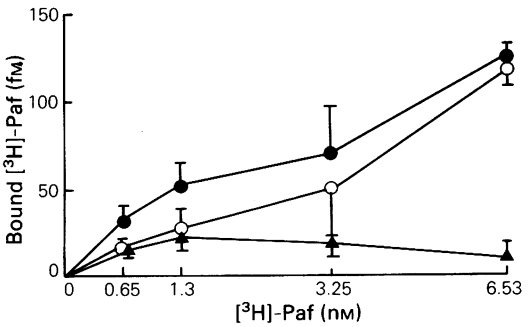

Figure 3 [^{3}H]-Paf binding to intact human platelets in the absence or presence of 2 μM WEB 2086. Saturable specific Paf binding (▲) was calculated in fmol from the difference of total (●) and non-specific binding (○). In this and following figures, values without platelets are subtracted from values in the presence of platelets and are means of 3 experiments with s.d. shown by vertical bars.

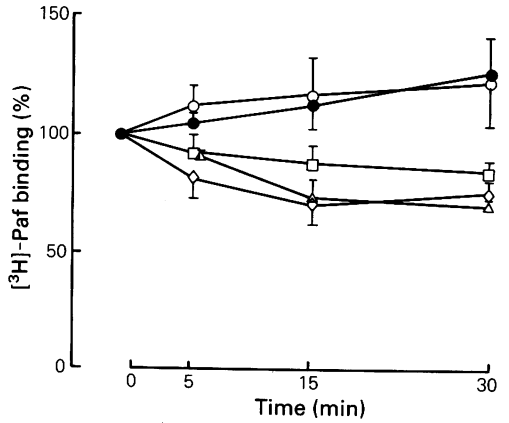

Figure 4 Displacement of platelet-bound [^{3}H]-Paf by increasing concentrations of WEB 2086 (4 ○, 40 □, 400 △, 2000 nM ◇, final concentrations) or vehicle (●, 0.2% ethanol, v/v). WEB 2086 was added to labelled platelets at time zero and platelet-bound radioactivity at various times thereafter was calculated as the percentage of platelet-bound radioactivity at time zero without WEB 2086.

aggregation was obtained with the antagonist versus 66% in the presence of vehicle (one experiment representative of three). WEB 2086 shifted the Paf dose-response curve rightwards in a parallel manner (Figure 2). The pA_2 was 7.9 with a slope of 0.81 and a correlation coefficient of 0.95 (Figure 2 inset).

WEB 2086, 2098 and 2105 exhibited similar inhibitory potencies on Paf-induced [^{14}C]-5-HT release as on platelet aggregation. The IC_{50} values for WEB 2086, 2098 and 2105 were 26.9 ± 25.0 nM, 30.3 ± 1.5 nM and 3.6 ± 4.0 μM, but could not be calculated for WEB 2118 (means ± 1 s.d. of three experiments).

[^{3}H]-*Paf binding to platelets in the presence of hetrazepines*

WEB 2086, 2098 and 2105 inhibited binding of 0.065 nM [^{3}H]-Paf in a concentration-dependent manner, with IC_{50} values of 26 ± 6 nM, 40.3 ± 3.5 nM and 1.76 ± 0.55 μM respectively (Figure 1b). At 0.065 nM nearly all [^{3}H]-Paf was specifically bound to platelets and the non-specific binding in the presence of an excess of WEB 2086 and 2098 was low. At concentrations up to 2 μM WEB 2118 failed to inhibit [^{3}H]-Paf-binding to platelets. There was a significant correlation between the IC_{50} values of the hetrazepines for inhibition of aggregation and inhibition of [^{3}H]-Paf binding when data were analysed by linear regression (Figure 1 inset).

Both total and non-specific binding of [^{3}H]-Paf in the absence and presence of WEB 2086 increased

with Paf concentration but did not reach a plateau (Figure 3). WEB 2086 indicated a saturable reversible component to Paf binding accounting for a maximum of 28.3 ± 3.7 fmol [^{3}H]-Paf per 5×10^7 platelets.

After 15 min preincubation of platelets with 0.65 nM [^{3}H]-Paf, platelet-bound label could be partially displaced by WEB 2086 in a concentration- and time-dependent manner (Figure 4). The displacement induced 30 min after the addition of 40–2000 nM WEB 2086 is shown in Figure 5. No additional displacement was obtained when 2 μM WEB 2086 was added to platelets either with or without 50 nM unlabelled Paf (Figure 5). At 40 nM WEB 2098 induced a moderate displacement, but similar concentrations of WEB 2105 and WEB 2118 failed to displace platelet-bound [^{3}H]-Paf (Figure 5).

Lack of metabolism of [^{3}H]-Paf in the presence of platelets

Platelets were incubated at 20°C or 37°C for 30 min with 0.65 nM [^{3}H]-Paf in the absence or presence of 40 nM WEB 2086. In all cases only intact Paf was identified by h.p.l.c. after extraction of filter-bound platelets (Figure 6).

Binding and metabolism of Paf to endothelial cells

WEB 2086 inhibited total [^{3}H]-Paf binding to endothelial cells in a concentration-dependent manner (Figure 7). In three experiments WEB 2086 inhibited the increase of cytosolic calcium in human endothelial cells triggered by a submaximal concentration of Paf (100 nM). After 3 min preincubation of cells with the antagonist, the WEB 2086 IC_{50} value was 23.1 ± 2.9 nM. After incubation of confluent endothelial cells with 0.65 nM [^{3}H]-Paf for 30 min only

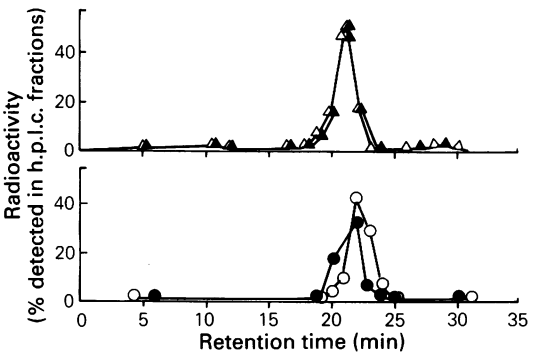

Figure 6 Lack of [^{3}H]-Paf metabolism in the presence of platelets and hetrazepines. Experiments were conducted in the absence (● ▲) or presence (○ △) of 40 nM WEB 2086 for 30 min at 37°C (▲ △) or 20°C (● ○). The figure shows 1 experiment representative of 3.

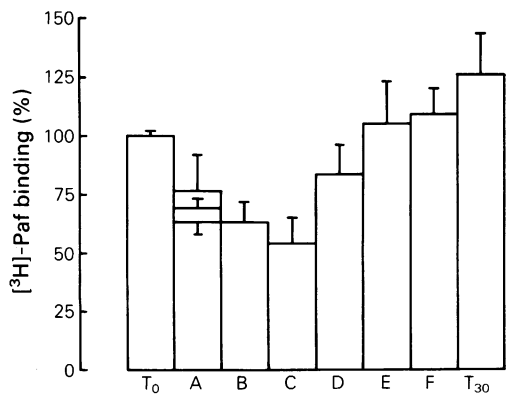

Figure 5 Displacement of platelet-bound [^{3}H]-Paf (0.65 nM) by WEB 2086. Platelets were preincubated for 15 min with 0.65 nM [^{3}H]-Paf alone (T_0). Hetrazepines were then added for 30 min before platelets were separated by vacuum filtration and binding measured: (A) from top to bottom 40, 1000, 2000 nM WEB 2086 in the absence of unlabelled Paf; (B) in the presence of 2000 nM WEB 2086 and 50 nM unlabelled Paf; (C) 50 nM unlabelled Paf alone; (D) WEB 2098; (E) WEB 2105; (F) WEB 2118, all at 40 nM; T_{30}, vehicle alone after 30 min incubation (T_{30}).

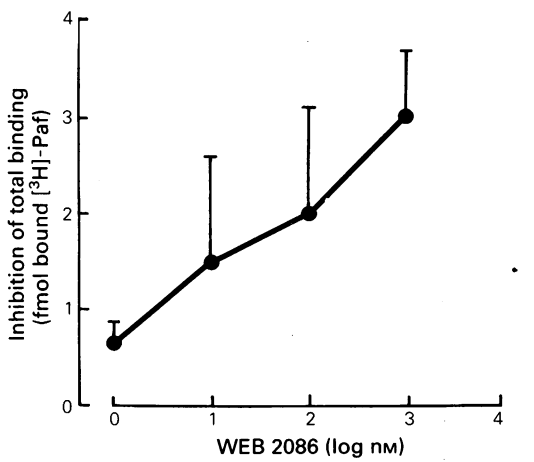

Figure 7 Concentration-dependent inhibition by WEB 2086 of total [^{3}H]-Paf binding to confluent endothelial cells. After 30 min incubation of endothelial cells with increasing concentrations of WEB 2086 (1–1000 nM) in the presence of 0.65 nM [^{3}H]-Paf, cells were detached and filtered. The inhibitory effect of WEB 2086 (●) on [^{3}H]-Paf binding to endothelial cells was calculated in fmol per 5.2 ± 0.15 × 10^5 cells. Values without cells were subtracted from filter-bound radioactivity in the presence of endothelial cells and are means of three experiments; s.d. shown by vertical bars.

$1.4 \pm 0.9\%$ and $3.4 \pm 3.4\%$ [^{3}H]-lyso-Paf and AAGPC were detected respectively.

Discussion

This study demonstrates that WEB 2086 inhibits platelet aggregation and Paf binding to platelets and endothelial cells in a concentration-dependent manner. The functional relevance of putative Paf receptors is supported by the fact that inhibition of platelet aggregation correlated closely with inhibition of Paf binding. The lack of inhibition of ADP-induced platelet aggregation by hetrazepines supports their specificity as described before with ADP and arachidonic acid (Casals-Stenzel, 1987a and our unpublished data).

Saturation of the inhibitory effect of WEB 2086 indicates its competition for a limited number of Paf binding sites in platelets. Similar results were obtained previously with unlabelled Paf, but not with its synthetic enantiomer (Korth & Benveniste, 1987). Platelets did not degrade Paf irrespective of whether antagonists were present, consistent with our observations with Ginkgolides (Korth & Benveniste, 1987; Korth *et al.*, 1988b). Competitive binding studies were performed at low [^{3}H]-Paf concentrations where nearly all [^{3}H]-Paf is specifically bound to putative Paf receptors. Platelet aggregation IC_{50} values of the hetrazepines correlated closely with their IC_{50} values versus Paf binding, sharing the same linear regression line with the Ginkgolides (Korth *et al.*, 1988b). Thus the same direct competitive inhibition which was previously demonstrated for Ginkgolides applies to hetrazepines. Substitution in the 9-position of the hetrazepine molecule modulates the inhibitory potency since WEB 2086 and WEB 2098 inhibited platelet aggregation and [^{3}H]-Paf binding at lower concentrations than WEB 2105 and WEB 2118 analogues (this article and Weber *et al.*, 1986).

In the aggregation studies WEB 2086 caused a rightward parallel shift with a slight depression in the maximum of the Paf dose-response curve at a concentration of 88 nM WEB 2086 (see Figure 2). We were not able to exclude an interaction between Paf, hetrazepines and albumin (our unpublished data). Therefore the same experiments were performed in the absence of albumin and using rabbit platelets which aggregate at lower Paf concentrations than human platelets. Rabbit platelets reached a maximal response to 1 μM Paf even in the presence of 88 nM WEB 2086 (unpublished results). The latter results are in agreement with those in the literature (Stewart & Dusting, 1988).

Association and dissociation binding studies (displacement) were performed since association studies alone cannot exclude allosteric effects or non-competitive receptor alterations. WEB 2086 displaced saturated platelet-bound [^{3}H]-Paf in a concentration- and time-dependent manner. No additive effects on Paf displacement were observed when WEB 2086 was incubated together with unlabelled Paf. The latter experiments favour a direct competitive hetrazepine/Paf receptor interaction, although more analysis would be helpful to exclude the presence of allosteric effects.

WEB 2086 also inhibited the binding of [^{3}H]-Paf and the Paf-mediated cytosolic calcium increase in cultured human vein endothelial cells. This might be of interest for further pharmacological and clinical studies since Paf binding sites in endothelial cells are different from those described in platelets (Korth *et al.*, 1987). Casals-Stenzel described an inhibitory effect of WEB 2086 on Paf-mediated vascular and bronchial effects in the isolated perfused rat lung even in the absence of blood cells indicating that endothelial cells may mediate pathophysiological effects of Paf (Casals-Stenzel *et al.*, 1987a).

Given the present results, it seems of interest to investigate hetrazepines in clinical studies since WEB 2086 is a very potent antagonist of Paf-induced effects in endothelial cells and platelets. The close correlation between inhibition of Paf binding and of platelet aggregation further emphasizes the importance of Paf receptors in the onset of the biological effects of this mediator.

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